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(54) Title: ACIDIFIED NITRITE AS AN ANTIMICROBIAL AGENT

(57) Abstract

The invention relates to the use of acidified nitrite as an antimicrobial agent and describes a dosage form for use in the treatment of bacterial, viral or fungal conditions. The dosage form may be in any pharmaceutically acceptable carrier means and comprises an acidifying agent adapted to reduce the pH at the environment. Amongst the many potential applications for the invention, the inventive composition has been shown to be particularly effective as an animal feed supplement, and as an agent for sterilising objects. Compositions and methods of use for these applications are described.

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ACIDIFIED NITRITE AS AN ANTIMICROBIAL AGENT

The present invention relates to acidified nitrite as an antimicrobial agent.

Although nitrite has been used as a preservative for food for many years the mechanisms by which it kills microorganisms has not been elucidated. We have now found that nitrite in low concentration is effective in reducing the populations of bacteria, fungi and viruses on the animal body when pH is below 4. We believe that this mechanism is used by mammals to destroy swallowed microorganisms.

An active entero-salivary circulation in man provides a continuous flow of nitrate into the mouth where it is rapidly reduced to nitrite by bacteria on the tongue. The effect of salivary nitrate excretion is to provide a precursor for the generation of nitrogen oxides by the break down of the nitrite.

In brief we have found that exposure of a yeast, Candida albicans and the bacterium E coli to concentrations of nitrite in saliva together with acid conditions similar to those found in the stomach for one hour caused a dose-dependent reduction in their survival. It is apparent therefore that the generation of nitrogen oxides and/or nitrous acid in the mouth and in the gastrointestinal tract, particularly the upper gastrointestinal tract, from acidified nitrite is preventative of microbial infection.

In the mouth bacteria rapidly reduce nitrates to nitrites. Once swallowed the acid conditions of the stomach protonate the nitrite to form nitrous acid (pKa approx 3.5). The nitrous acid in turn dissociates to form oxides of nitrogen as shown below.

$$NO_2^- + H^+ = HNO_2 \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots \quad (1)$$

$2HNO2 = H_2O + N_2O_3$ ..... (2)

$N_2O_3 = NO + NO_2$ ..... (3)

Endogenous and dietary nitrate is actively concentrated by salivary glands to more than 10 times the concentration in plasma and secreted in saliva. Thus the saliva provides a continuous source of nitrate to the upper gastrointestinal tract. Oral conversion of nitrate to nitrite is rapid and is restricted to the surface of the tongue in man and to the posterior third of the tongue in the rat.

The function of the entero-salivary circulation of nitrate is not known but it may well be that gastric acid by itself is not always sufficient to destroy many ingested micro-organisms and that the primary role of salivary nitrate secretion and conversion to nitrite is as a precursor for nitrogen oxides in the lumen of the stomach which will kill swallowed micro-organisms.

The above identified mechanism is also applicable to the destruction of micro-organisms on the skin. For example athlete's foot or tidea pedis.

We have found that nitrite at concentrations of up to 4% in an inert carrier cream or ointment when mixed with an organic acid such as salicylic acid reacts to produce oxides of nitrogen which are effective in killing infectious organisms on the skin including fungi, yeast, bacteria and viruses. The combination of nitrite and acid causes mild erythema (redness) of the skin due to release of nitric oxides but this causes no significant inflammation.

The above identified mechanism is also useful in the sterilisation of objects such as dentures by utilising a sterilizing nitrate solution. Conventional solutions which are effective in sterilising dentures often taste unpleasant due to chlorine-based disinfectants. A combination of nitrite and

acid results in a antimicrobial solution which has little or no taste. Other objects such as contact lenses may be sterilised in the same way.

Gastroenteritis continues to be a major problem in rearing pigs and other farm animals. Enteropathogenic Escherichia coli (especially those bearing the K88 antigen) are particularly implicated. Although gastric acidity is thought to be one of the main host defence systems which provides a barrier to orally-acquired infection, this is clearly ineffective in preventing organisms from reaching the more distal intestine in these animals.

According therefore to a first aspect of the present invention there is provided a dosage form for the treatment of bacterial, virus, or fungal conditions which comprises:-

a pharmaceutically acceptable acidifying agent,

a pharmaceutically acceptable source of nitrite ions or a nitrate precursor therefor, and

a pharmaceutically acceptable carrier or diluent, wherein the acidifying agent is adapted to reduce the pH at the environment of use to below pH4. Preferably the acidifying agent is an organic acid, for example salicylic acid or ascorbic acid. The precursor for the nitrite ion may be an alkaline metal or alkaline earth metal nitrate capable of conversion to a nitrate by enzymic action.

The pharmaceutical acceptable carrier or diluent may be an inert cream or ointment. In a particularly preferred form of the invention the acidifying agent and the source of nitrite ions or precursor therefor are separately disposed in said cream or ointment for admixture to release nitrite ions at the environment of use.

Alternatively an acid composition may be presented for administration in tablet or liquid form.

In a further aspect of the invention there is provided a method of sterilising an object which method comprises the steps of

1) preparing a pharmaceutically acceptable acidifying agent and a pharmaceutically acceptable source of nitrite ions,

2) mixing said acidifying agent with said source of nitrite ions in a liquid carrier or diluent in contact with said object thereby to reduce the pH to below 4 while causing said sterilant nitrite ions to sterilize said object.

In a further form of the invention there is provided a sterilant composition comprising a pharmaceutically acceptable acidifying agent,

a pharmaceutically acceptable source of nitrite ions or a nitrate precursor therefor,

and a pharmaceutically acceptable carrier or diluent therefor wherein the acidifying agent is adapted to reduce the pH at the environment of use to below pH4.

In a still further form of the invention there is provided an animal feed supplement comprising a pharmaceutically acceptable acidifying agent and,

a pharmaceutically acceptable source of nitrite ions or a nitrate precursor therefor, in an amount sufficient to produce a beneficial anti-bacterial pharmalogical effect, but insufficient to produce adverse action in the target animal.

The acidifying agent may be salicylic or ascorbic acid as above, and the source of nitrite ions or nitrate precursor therefor may be in an inorganic nitrate as set forth above. Where the animal is the pig, the supplement should be included in an amount sufficient to ensure that each adult animal will receive a balanced dose of between 0.3 to 5.0 g/day and preferably about 1 g/day.

The invention will now be described, by way of illustration

only, with reference to the following examples and figures accompanying the specification.

Figure 1 shows a diagram indicative of the effect of exposure to nitrate and differing hydrogen ion concentrations on the survival of C albicans where the vertical axis is the optical density in absorbance units and the horizontal axis is the pH.

Figure 2 shows growth curves of E coli following exposure to acid alone or acid with a nitrite where the vertical axes are optical density in absorbance units and the horizontal axes are time in hours.

Figure 3 shows growth curves of E coli following exposure to pH3 in various nitrite concentrations where the vertical axis shows the optical density in absorbance units and the horizontal axis is time in hours.

Figure 4 shows the generation of nitric oxide from sodium nitrite at different levels of acidity where the vertical axis is the nitric oxide concentration (nM) and the horizontal axis is Ph.

EXAMPLE 1

With reference to Figure 1 a single colony of C albicans was used to inoculate an overnight culture in Sabouraud's broth. 10μl of this broth was added to 940μl of a citrate/phosphate buffered Sabouraud's broth to which was added sodium nitrite (50μl; final concentration 250μM) or distilled water as a control. After one hour incubation at 37°C, 10μl was removed and cultured in 190μl standard Sabourauds broth with continual agitation (Gallenkamp orbital incubator) in a 96-well microtitre plate at 37°C. Growth was monitored by measurement of optical density at 570nm at regular time intervals. The results are a mean of 16 separate experiments.

The effect of exposure to nitrite and differing hydrogen ion

concentrations on the survival of C albicans is shown in Figure 1. The open bars show the growth of C albicans measured by the optical density method following exposure to acid alone for 1 hour, while the closed bars show growth following exposure to acid and 250μM sodium nitrite. There is a significant difference from the control at $p>0.05$ (Mann-Whitney U test). It is apparent therefore that the incubation of C albicans in acid alone for one hour had little effect on the number of viable organisms subsequently grown, whereas in contrast the addition of sodium nitrite at 250μM incrementally killed C albicans as the pH was reduced to below 4. The nitrite was in fact effective in eliminating C albicans at pH 1 at all concentrations above 250μM (data not shown). 5nN nitrite killed C albicans at up to pH5. It is significant that a random sample of 10 laboratory personnel on a normal diet had fasting salivary nitrite which varied from 23 to 220μM (mean 114μM) rising to 409 to 1890μM (mean 1030) 45 minutes after ingestion of 200mg potassium nitrate solution.

EXAMPLE 2

Figure 2 shows growth curves of E coli following exposure to acid alone (open symbols) or acid and 250μM nitrite (closed symbols). Growth was significantly ($p<0.05$) impaired at pH 2,3 and 4 in the presence of nitrite compared with control.

The same methods were used as in Figure 1 except E coli (strain NCTC 10418 grown on MacConkey's agar) was used and nutrient both (Oxoid CM1) was used in place of Sabouraud's broth. The results shown in Figure 2 are a mean of 20 experiments. As can be seen from Figure 2 E coli is more susceptible to acid than C albicans. Nevertheless exposure to pH 2 for one hour does not kill all the organisms as there is significant growth in the nutrient broth. At pH3 many organisms survive. The addition of 250μM nitrite to the exposure medium eliminates E coli at pH2 and significantly reduces the viability of this organism at pH3 and pH4. Nitrite at this concentration had no effect above pH4.

EXAMPLE 3

Figure 3 shows growth curves of E coli following exposure to pH3 in various nitrite concentrations (10-1000μM final concentration). The methods are those as for Figure 2. Figure 3 shows that there is a direct relationship between the toxic effects of nitrite on E coli and nitrate concentration at pH3. Even 10μM had a discernable effect whereas 1mM killed E coli completely.

EXAMPLE 4

Figure 4 shows the generation of nitric oxide from sodium nitrite (as μM) at different acidities. Conditions were the same as those used for the exposure of organisms in Figure 1. In particular nitrite was added to citrate/phosphate buffer to achieve final concentrations shown in Figure 4. Nitric oxide concentrations in the buffer were measured by a nitric oxide sensitive meter (ISO-NO, World Precision Instruments) connected to a Maclab acquisition system and Macintosh computer. Measurements were recorded continually and readings were taken at 2 minutes when nitric oxide concentration had reached a steady state. Figure 4 shows the release of nitric oxide as a result of reducing pH. Nitric oxide, which we have shown is generated under experimental conditions in Figure 4 readily diffuses through cell membranes and has a high affinity for iron-sulphur containing respiratory enzymes and damages bacterial DNA. When produced enzymatically by activated leucocytes, nitric acid will kill Leishmania sp., Staphylococcus sp., Francisella sp. and Microbacterium as well as C albicans. Reaction with superoxide under acid conditions may additionally produce highly reactive hydroxyl radicals.

EXAMPLE 5

In a study to investigate the effect of a combination of salicylic acid at 2% w/w and sodium nitrite at 2% w/w in 9 patient volunteers with microbiologically proven fungal infection of the feet, application of the treatment produced a microbiological cure in all but one patient after 2 weeks

of therapy. The symptom score (derived from a scoring system which measures erythema, vesicles, pustules, desquamation, encrustation and pruritus) decreased from a mean of 7 before treatment to a mean of 2 following treatment.

EXAMPLE 6

Investigation of the use of nitrate or nitrite administered topically in the mouth in the form of toothpaste, mouthwash or other orally acceptable vehicle to reduce the number of caries-producing organisms in dental plaque and to treat to prevent infection with C albicans or other harmful organisms showed such application to be effective.

The observation that oxides of nitrogen produced non-enzymatically from nitrite under conditions simulating those in the stomach kills C albicans and E coli extends these observations to the intestinal tract. E coli is closely related to Salmonella, Shigella and other pathogenic enterobacteria; all important causes of gastroenteritis in the mammal.

These results provide a rationale for active secretion of nitrate by the salivary glands. Nitrate itself is a innocuous precursor which only produces microbiocidal species when converted to nitrite and subjected to acid conditions. It is possible that Lactobacilli sp. transiently produce sufficient acid in the mouth after a carbohydrate meal to control the growth of oral pathogens but clearly a moderate intake of nitrate may be a desirable prerequisite in any contaminated environment despite any potential as a precursor of nitrosamines.

Further the production of intestinal nitrogen oxides may be inadequate if the oral flora which convert nitrate to nitrite are suppressed following therapy with broad-spectrum antibiotics. Similarly if gastric acid production is reduced,

or if nitrate intake, which is largely dependent on leafy vegetables, is low this protective mechanism will be impaired. These are precisely the situations which predispose to oral and intestinal infections.

Whereas the foregoing study has concentrated on C albicans and E coli and the other organisms mentioned, it may also be important for providing protection from other serious gut pathogens which when swallowed may cause duodenal ulceration, for example Helicobacter pylori, amoebic dysentery and chronic intestinal parasitism. Accordingly the invention provides a dosage form for the treatment of bacterial, viral or fungal conditions, a method of sterilising an object, and a composition therefor.

The above also suggests an inexpensive and simple means of prevention of gastroenteritis in farmed pigs by modification of dietary nitrate intake without the use of antibiotics.

CLAIMS

1. A dosage form for treatment of bacterial, viral or fungal conditions which comprises;

a pharmaceutically acceptable acidifying agent,

a pharmaceutically acceptable source of nitrite ions or a nitrate precursor therefor,

and a pharmaceutically acceptable carrier or diluent therefor, wherein the acidifying agent is present in an amount sufficient to reduce the pH at the environment of use to below pH4.

2. A dosage form according to claim 1 wherein the acidifying agent is an organic acid.

3. A dosage form according to claim 2 wherein the acidifying agent is salicylic or ascorbic acid.

4. A dosage form according to any preceding claim wherein the nitrate precursor is an alkali metal or alkali earth metal nitrate.

5. A dosage form according to any preceding claim wherein the pharmaceutically acceptable carrier is disposed in an inert cream or ointment, and wherein said acidifying agent and said source of nitrite ions is separately disposed in a respective cream or ointment for admixture to release nitrate ions at the intended environment of use.

6. A dosage form according to any of claims 1 to 4 in tablet or liquid form.

7. A method of sterilising an object which method comprises the steps of:-

1) preparing a pharmaceutically acceptable acidifying agent and a pharmaceutically acceptable source of nitrate ions or a nitrate precursor therefor,

2) admixing said acidifying agent with said source of nitrite ions in a liquid carrier or diluent in contact with said object, thereby to reduce the pH to below 4 to release sterilant nitrite ions to sterilize said object.

8. A method according to claim 7 wherein said acidifying agent is an organic acid.

9. A method according to claim 8 wherein said organic acid is a salicylic acid.

10. A method according to any of claims 7 to 9 wherein said precursor is an alkali metal or alkali earth metal nitrate.

11. A sterilant composition comprising a pharmaceutically acceptable acidifying agent,

a pharmaceutically acceptable form of nitrite ions or a precursor therefor,

and a pharmaceutically acceptable carrier or diluent therefor, wherein the acidifying agent is adapted to reduce the pH at the environment of use to below 4.

12. An animal feed supplement comprising a pharmaceutically acceptable acidifying agent, and a pharmaceutically acceptable source of nitrite ions or a nitrate precursor therefor, in an amount sufficient to produce a beneficial anti-bacterial effect but insufficient to produce an adverse reaction in a target animal.

13. An animal feed supplement according to claim 12 wherein the acidifying agent is selected from salicylic or ascorbic acid.

14. An animal feed supplement according to either of claims 12 or 13 wherein the source of nitrite ions is an inorganic nitrate.

15. An animal feed supplement according to claim 14 wherein the feed supplement is adapted for the pig, and the inorganic nitrate is present in the feed in an amount sufficient to provide an adult pig with about 1 g/day.

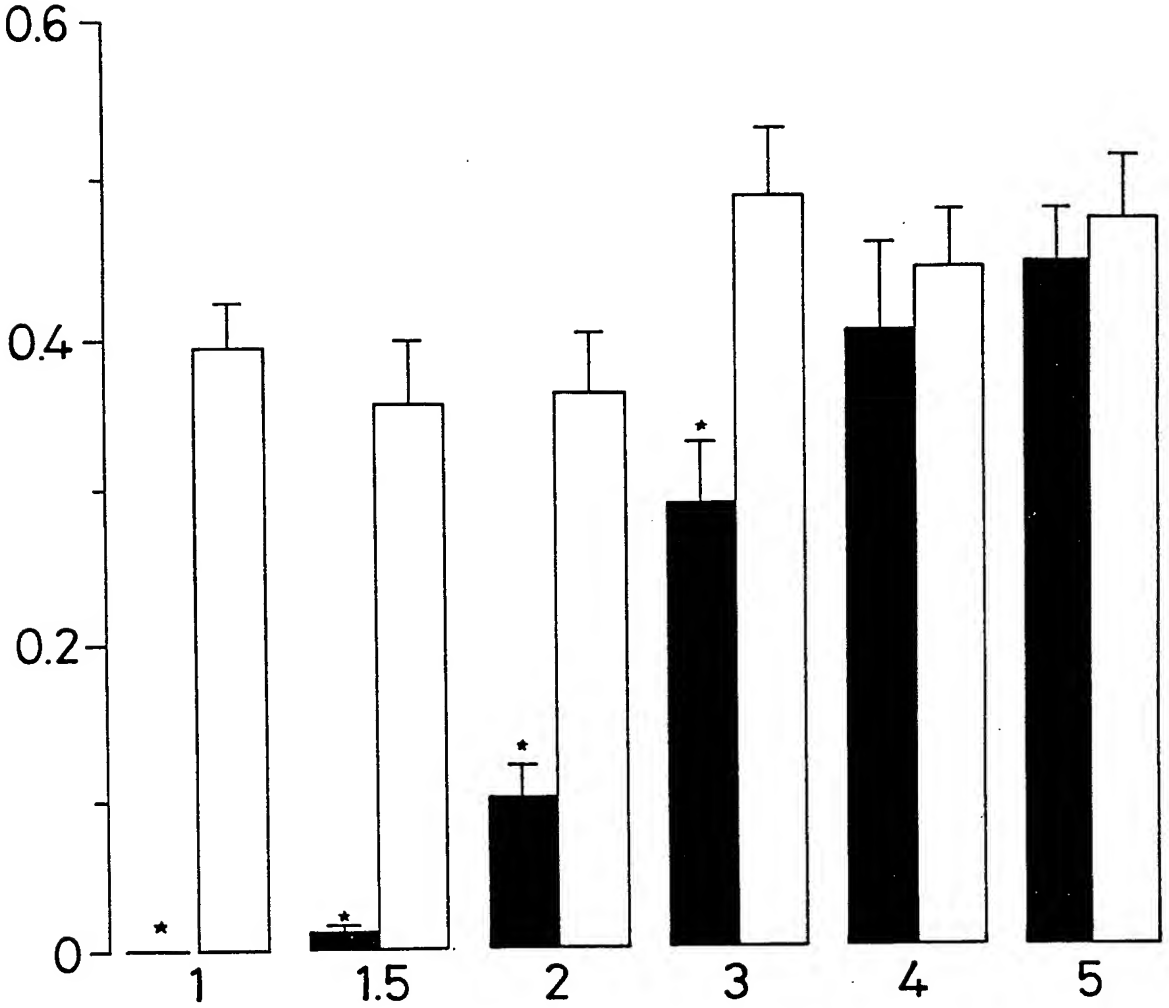


FIG. 1

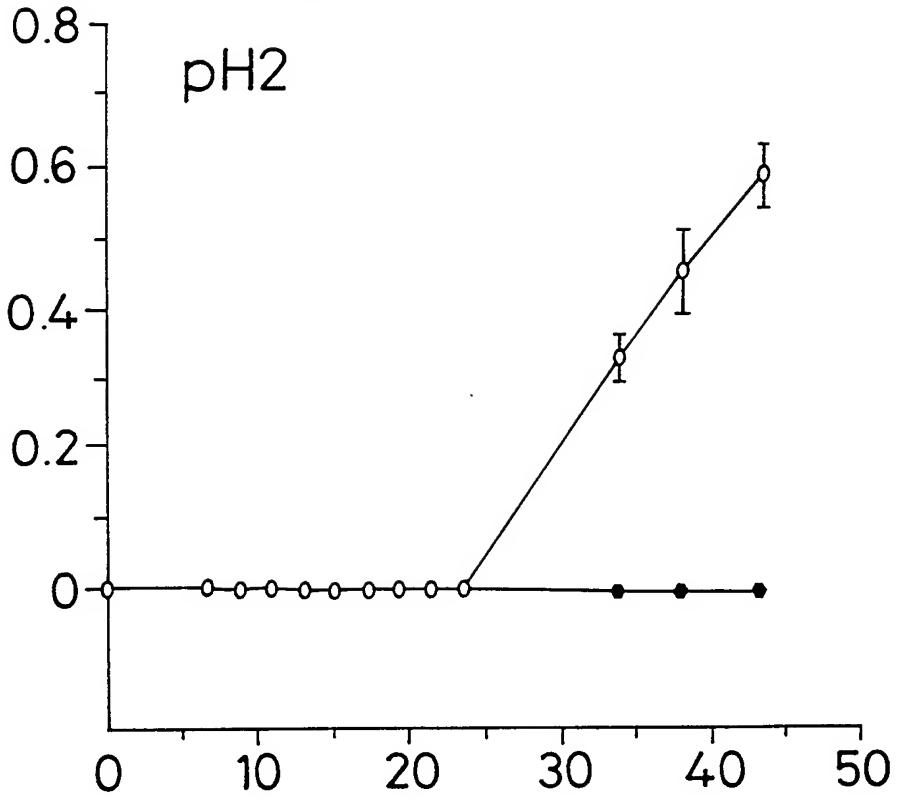
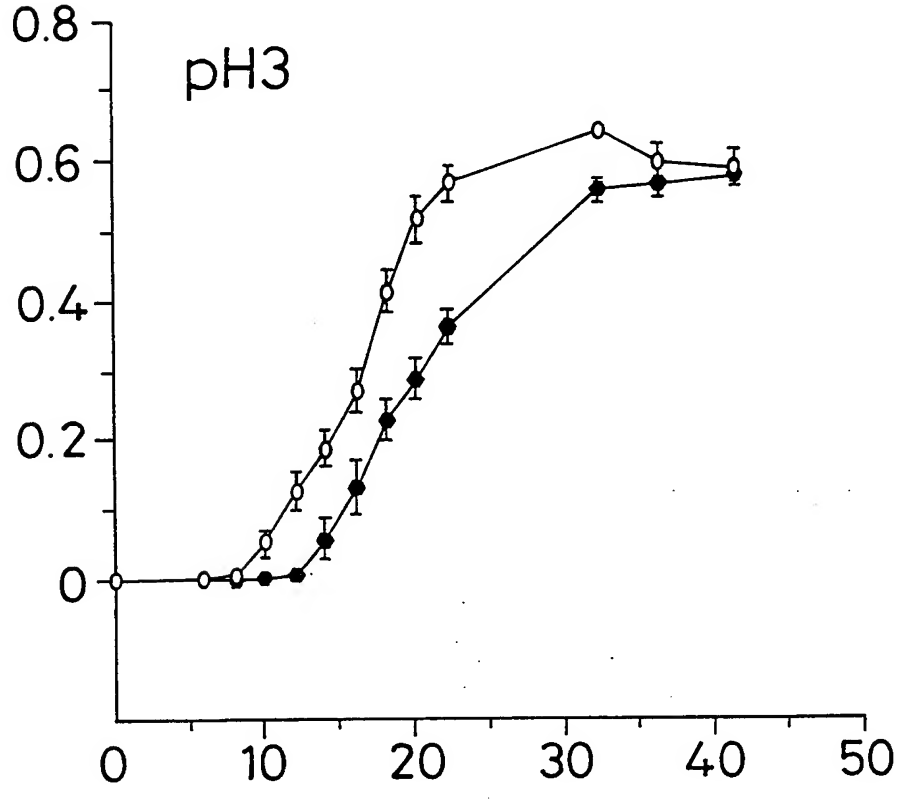


FIG. 2



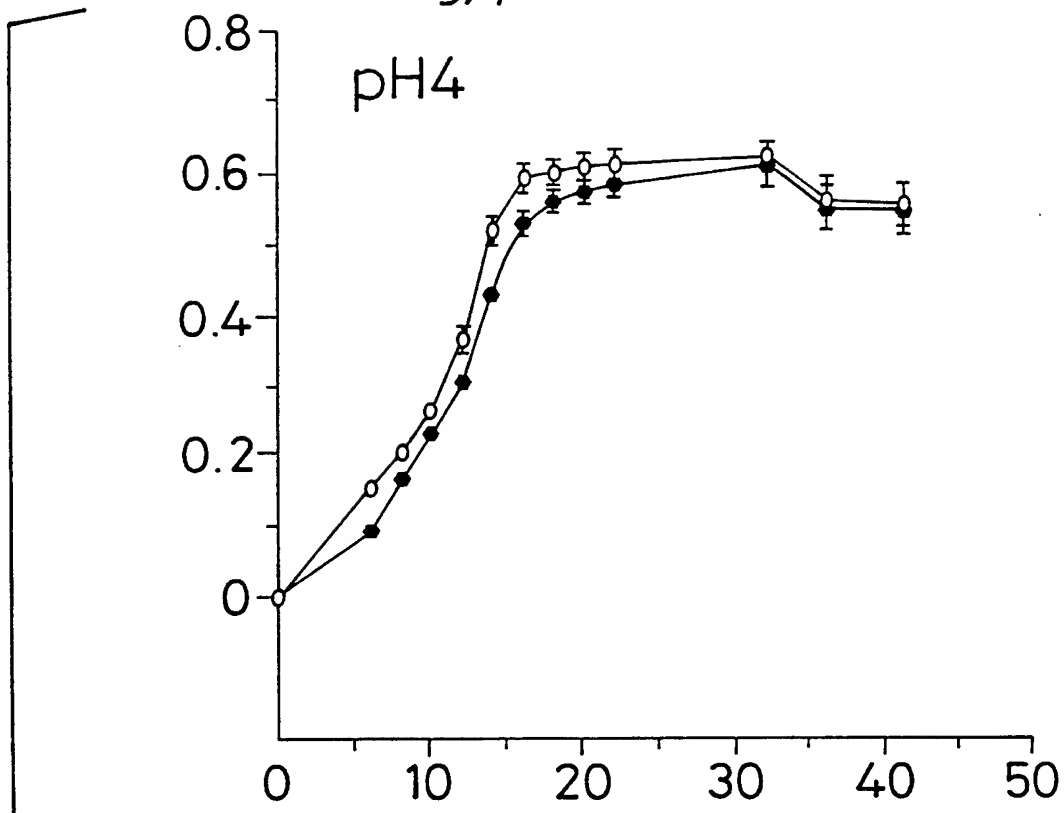
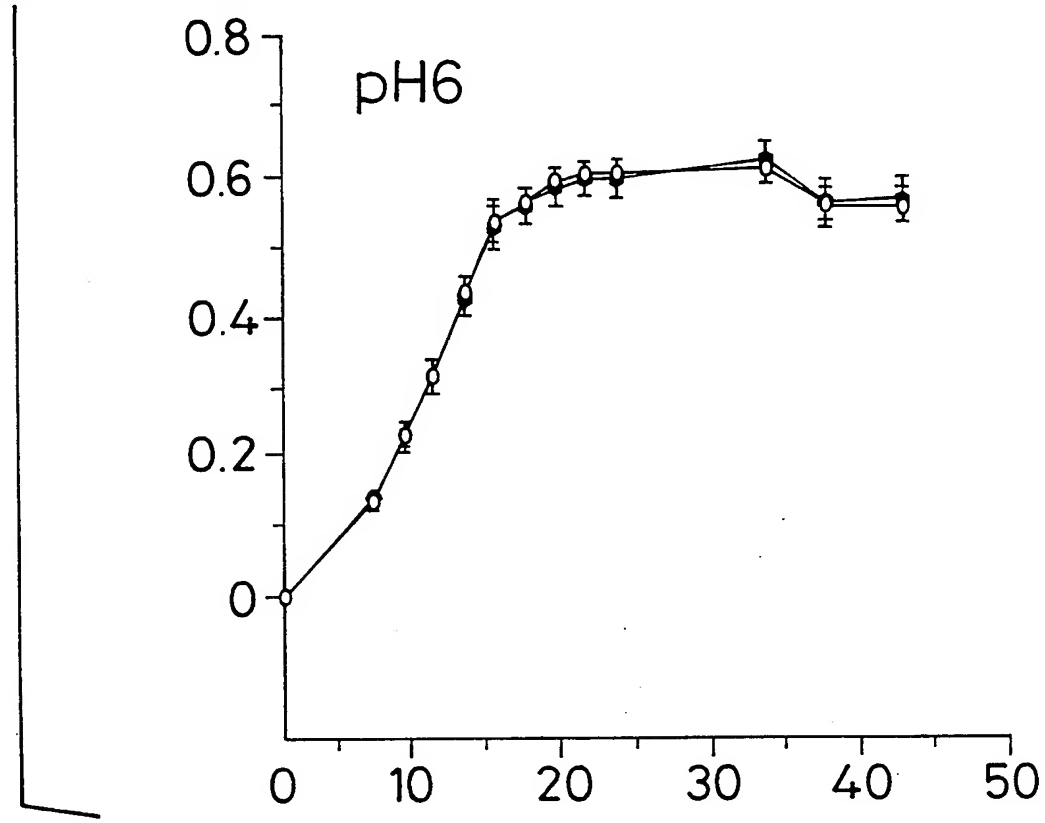


FIG. 2 (Continued)



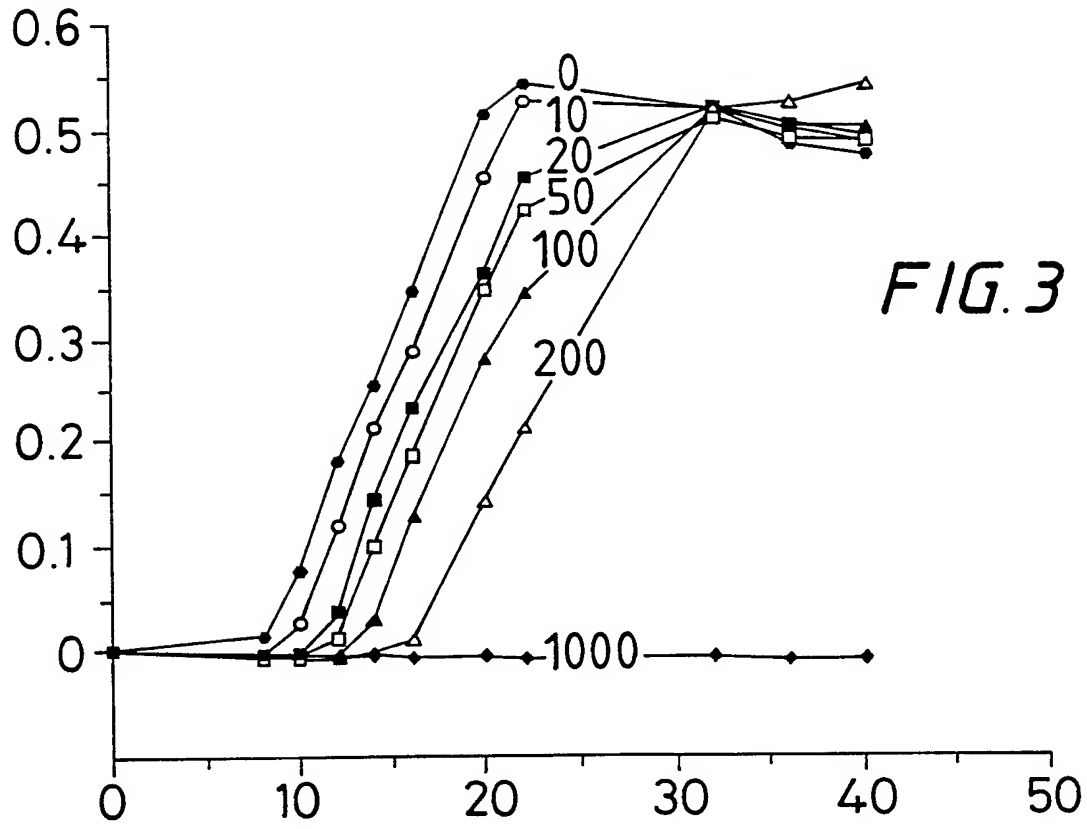

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FIG. 3

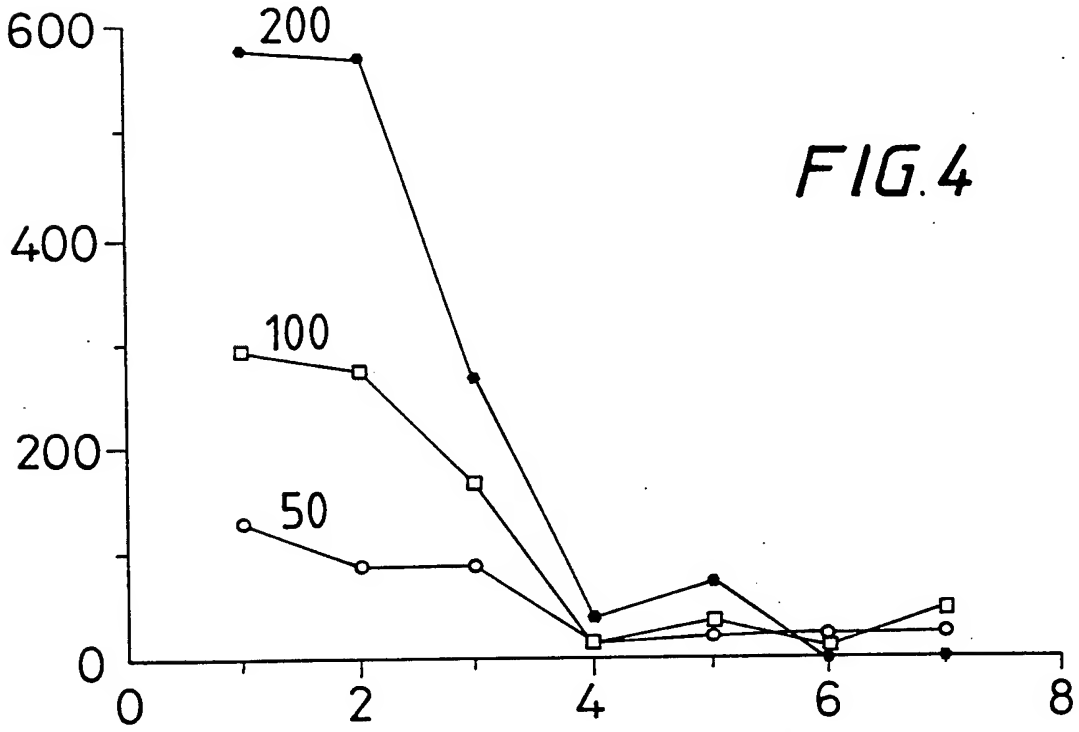
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FIG. 4

INTERNATIONAL SEARCH REPORT

Int. ional Application No
PCT/GB 95/00338

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 A61K33/00 A61K47/12

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	DATABASE WPI Week 8426 Derwent Publications Ltd., London, GB; AN 84-161228 & JP,A,59 085 278 (AGENCY OF IND. SCI. TECH.) , 17 May 1984 see abstract	1-3
X	DATABASE WPI Week 8731 Derwent Publications Ltd., London, GB; AN 87-217091 & JP,A,62 142 559 (SHOKO KK) , 25 June 1987 see abstract	7,11
A	US,A,4 191 750 (HODOSH) 4 March 1980	

☐ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

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1

Date of the actual completion of the international search	Date of mailing of the international search report
30 May 1995	16.06.95

Name and mailing address of the ISA	Authorized officer
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Information on patent family members

Int. ional Application No
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Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US-A-4191750	04-03-80	NONE	